Use of BRAF V600E as a Molecular Marker in Aggressive Colorectal Cancer

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ABSTRAK

Tujuan: *meneliti perbedaan imunoekspresi BRAF V600E di antara stadium Dukes pada karsinoma kolorektal.* ***Metode:*** *penelitian ini retrospektif, potong lintang, terhadap kasus karsinoma kolorektal masing-masing stadium Dukes A, B dan C sebanyak 15 kasus. Dilakukan pewarnaaan antibodi BRAF V600E terhadap potongan tipis massa tumor untuk mengevaluasi adanya protein BRAF V600E. Perbedaan proporsi imunoekspresi BRAF V600E di antara masing-masing stadium Dukes pada karsinoma kolorektal diuji menggunakan uji Chi-Square.* ***Hasil:*** *hasil pewarnaan imunohistokimia yang positif (positif sedang sampai positif kuat) pada stadium Dukes A, B, C adalah sebagai berikut: 1 kasus dari 15, 4 kasus dari 15 kasus dan 13 kasus dari 15 kasus. Pewarnaan imunohistokimia BRAF V600E yang positif lebih banyak ditemukan pada stadium Dukes C dan sangat bermakna (p<0,001) berdasarkan uji statistik Chi-Square.* ***Kesimpulan:*** *hasil pewarnaan imunohistokimia BRAF V600E dapat dipakai sebagai petanda karsinoma kolorektal yang lebih agresif.*

Kata kunci: *BRAF V600E, Dukes, imunoekspresi, karsinoma kolorektal.*

ABSTRACT

Aim: *to compare the immunoexpression of BRAF V600E among stage of colorectal cancer.* ***Methods:*** *this was a cross sectional, and retrospective study involving Dukes' stage A, B, and C colorectal carcinoma, each with 15 cases. Immunohistochemistry was performed in paraffin-embedded specimens of tumor mass for the assessment of BRAF V600E. The proportion differences of immunoexpression of BRAF V600E among Dukes'stage A, B, and C were tested using Chi-Square test.* ***Results:*** *the result of positive BRAF V600E immunoexpression (moderately to strongly positive) in Dukes'stage A, B, and C were found in 1 of 15 cases, 4 of 15 cases and 13 of 15 cases respectively. BRAF V600E immunoexpression was statistically significant more frequent in Dukes'stage C (p<0,001, Chi-Square test).* ***Conclusion:*** *positive BRAF V600E immunoexpression could be used as a marker of aggresive colorectal carcinoma.*

Key words: *BRAF V600E, Dukes, immunoexpression, colorectal carcinoma.*

INTRODUCTION

Colorectal carcinoma (CRC) is the term for colon or rectum malignant epithelial tumor.[1] Colorectal cancer (CRC) is an important global health problem.[2] Cancers of the colon and rectum are the third most common type worldwide.[3]

Colon cancer stage is the most important factor in predicting patient outcomes.[4] First staging classification-Dukes' stage is stage A, B, C, in which stage A tumours were confined to the rectal wall, stage B tumours breached extra-rectal tissues, and stage C tumours

had metastasised to lymph nodes. Treatment decisions are usually made in accordance with older Dukes classification schema. Primary colorectal carcinoma (CRC) treatment is surgery to remove the primary tumor. Adjuvant chemotherapy is a standard of care in stage III or Dukes' stage C patients while its role is less well established in stage II or Dukes' stage B.[5,6] Hence, other criteria for adjuvant therapy are needed. Molecular markers might prove to be better than clinical and histopathological criteria for therapy selection.[7]

The Ras/Raf/MEK/ERK mitogen-activated protein kinase (MAPK) pathway is a critical signal transduction pathway involved in many cancers, including colorectal cancer, and has been the target of therapeutic intervention in recent years.[8] Critical to progress are molecular tests identifying the underlying genetic causes of dysregulated cell proliferation, such as certain BRAF gene mutations causing constitutive MAPK signaling and transcription-mediated cell division and survival. Recently, BRAF mutation testing has been introduced into routine clinical laboratories because its role has become clearer in terms of effect on pathogenesis of CRC.[9] Ninety percent of BRAF mutations are accounted for by a thymine to adenine single-base change at position 1,799. This missense mutation, located in exon 15, results in a change at residue 600 that substitutes glutamine for valine (V600E).[10] The encoded protein has more than 10 times more kinase activity than its normal counterpart. Thus, BRAF V600E acts as an oncogene.[9] Activating mutations in BRAF have been reported in 5–15% of CRC.[11]

Currently, only molecular methods, such as polimerase chain reaction (PCR), and various sequencing technologies are available for assessing BRAF mutational status. Thus, analysis is limited to high complexity molecular testing facilities, requiring specialized equipment and proficient personnel. This can increase cost and turn-around time, affecting patient care. Immunohistochemistry is available routinely in most pathology laboratories and, frequently, at a lower cost.[12] An immunohistochemical approach for assessing BRAF V600E mutation in colorectal cancer and its relationship to Dukes'stage are evaluated to determine BRAF V600E status in colorectal cancer.

METHODS

This was cross sectional, retrospective study. We included Dukes' stage A, B, and C colorectal carcinoma each with 15 archival paraffin blocks of tumor mass at Department of Anatomical Pathology, Hasan Sadikin Hospital, Bandung, from 2011-2012 for immunohistochemistry staining. We chose the case by consecutive sampling method. This study was conducted with human ethics review committee approval.

Immunohistochemistry

All hematoxylin-eosin (H+E) cases were reviewed for Dukes'stage and one representative block of each tumor was selected, 4μm sections were cut, and immunohistochemistry was performed using the anti BRAF V600E antibody VE1(Spring Bioscience). The generation and validation of this antibody have been reported previously.[12] We used Labeled Streptavidin Avidin Biotin (LSAB) technique for immunohistochemical analysis.

The slides were heated in a 38°C oven for 30 min, deparaffinized in three changes of xylene for 5 min each, rehydrated in graded alcohols (90%, 80%, and 70%) for 5 min each, and rinsed in deionized water. Antigen retrieval was performed in sodium citrat buffer 10mM in a pressure cooker (BioCare Medical, Concord, CA) for 30 min. The slides were rinsed in deionized water, rinsed in phosphate buffer saline (PBS) pH 7,2, applied blocking serum for 10 min, rinsed in PBS. The primary antibody (BRAF V600E, clone VE1, Spring Bioscience) was applied for overnight at room temperature at a 1:200 dilution. The sections were gently rinsed in PBSx2 for 5 min each. Secondary antibody Trekkie universal link (Biocare Medical) was applied for 20 min and rinsed in PBSx2 for 5 min each. The sections were labeled by TrekAvidin (HRP) for 10 min and rinsed in PBS x2 for 5 min each. Antigen detection was performed using the Betazoid DAB as the chromogen. The slides were counterstained with hematoxylin Mayer for 2 minutes. The sections were gently rinsed in deionized water. The sections were dehydrated

in graded alcohols (70%, 80,% and 96%), cleared in xylene, and coverslipped. Positive controls were thyroid papillary carcinoma and negative controls were provided by omitting the primary antibody.

All H+E, and BRAF V600E-immunostained slides were reviewed by three pathologists independently (BSH, AHH, SS). The intensity of cytoplasmic tumor cell staining and the percentage of tumor cells stained were assessed. Slides that have different result interpretation were then viewed by these observers together to reach agreement on any discordant scores.

Scoring System

The expression of BRAF V600E was observed in the cytoplasm of tumor cells (**Figure 1–4**). Immunoexpression was judged on a semi-quantitative scale. The BRAF V600E expression was scored as percentage of positive neoplastic cells and intensity of immunoreactivity. The percentage scoring of immunoreactive (IR) tumor cells was as follows: 0 (0% IR cells), 1 (1–10% IR cells), 2 (11–30% IR cells), 3 (31–50% IR cells), and 4 (>50%). The staining intensity was scored as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). A final score was obtained for each case by multiplying the percentage and the intensity score. Therefore, four categories for BRAF V600E expression were assigned: negative (score 0), low (scores 1 and 2), and moderate (score 3 and 4) and high (score 6–12). The slides were assessed for cytoplasmic staining only.

Statistics

The data were evaluated by means of bivariate analysis Chi-square using SPSS for Windows version 20 (SPSS, Chicago, Ill., USA). Independent variable were BRAF V600E immunoexpression and dependent variable were Duke's stage CRC. The results expressed as $p<0.05$ were considered statistically significant.

RESULTS

A total of 45 cases of colorectal carcinoma were assessed in immunohistochemistry staining pattern. We selected 15 cases each Dukes' stage (A, B, C) with complete archive paraffin block and representative tumor mass and reviewed Dukes' stage from H+E slides. The clinical and pathological features are presented in **Table 1**.

Table 1. Characteristic patient colorectal carcinoma

Variables	Colorectal Carcinoma			Σ
	Dukes A	Dukes B	Dukes C	
Age				
- Mean: 52.7 year				
- Standard deviation: 12.8 year				
Sex				
- Man	8	11	6	25
- Woman	7	4	9	20
Histopathologic variant				
- Adenocarcinoma	11	10	13	34
- Adenocarcinoma with mucinous degeneration	0	1	0	1
- Mucinous adenocarcinoma	3	4	2	9
- Signet-ring cell carcinoma	1	0	0	1
Grade				
- Well	11	6	8	25
- Moderately	0	3	5	8
- Poorly	0	1	0	1
- Without grade	4	5	2	11

Briefly, the mean age was 52.7 years (Standard deviation = 12.8 year) and 25 of 45 cases were males. Most patient 34 of 45 cases presented with adenocarcinoma variant. **Figure 1–4** along with photomicrographs of representative staining patterns.

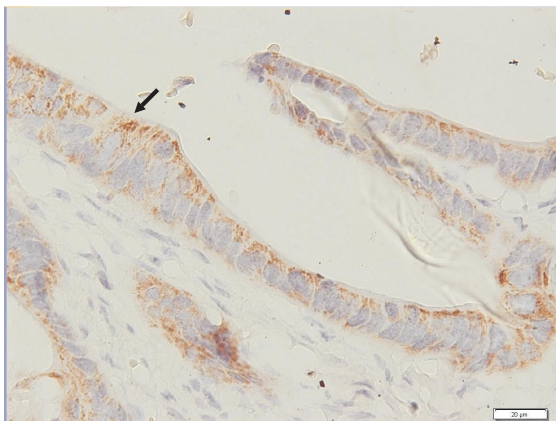

Figure 1. Strong intensity BRAF V600E immunoexpression (400x). Arrow show dark brown cytoplasm (=Strong Intensity)

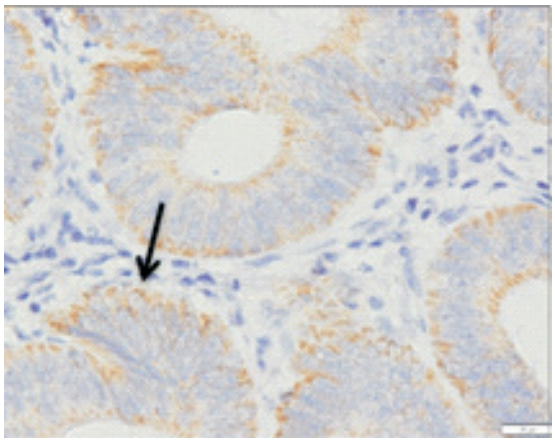

Figure 2. Moderate intensity BRAF V600E immunoexpression (400x). Arrow show brown cytoplasm (=Moderate Intensity)

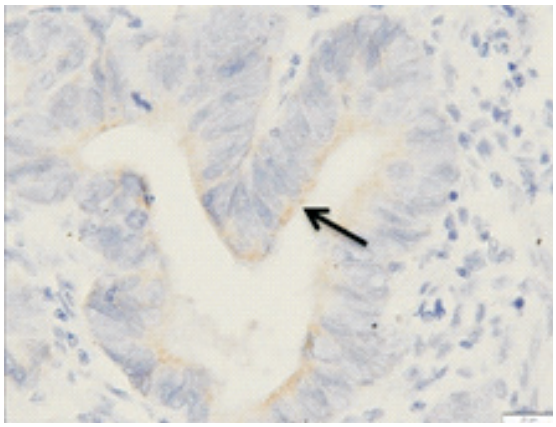

Figure 3. Weak intensity BRAF V600E immunoexpression (400x). Arrow show light brown cytoplasm (=Weak Intensity)

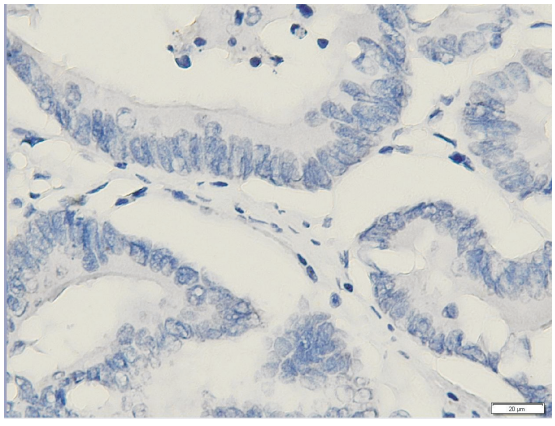

Figure 4. Negative BRAF V600E immunoexpression (400x)

BRAF V600E Immunoexpression in Dukes' Stage of CRC

BRAF V600E immunoexpressions were assesed for cytoplasmic staining only. Twenty-three of 45 CRCs were positive BRAF V600E immunoexpressions (7 cases of 45 cases with strongly positive, 11 cases of 45 cases with moderately positive, and 5 cases of 45 cases with weakly positive) and 22 of 45 CRCs were negative BRAF V600E immunoexpressions.

Table 2. Result of BRAF V600E immunoexpressions in Dukes' stage of CRC

Immunoexpression BRAF V600E	Stage (n)			Total (N)
	Dukes A	Dukes B	Dukes C	
Strongly positive	1	1	5	7
Moderately positive	0	3	8	11
Weakly positive	1	4	0	5
Negative	13	7	2	22
Total	15	15	15	45

Chi-Square test showed that the proportion of positive BRAF V600E immunoexpression was significantly higher in Dukes' stage C ($P < 0{,}001$). The results of BRAF V600E immunoexpression in three groups were sumarized in **Table 3**.

Table 3. BRAF V600E immunoexpression in Dukes' stage CRC

Immunoexpression BRAF V600E	Stage (n)			Total (N)
	Dukes A	Dukes B	Dukes C	
Moderately to strongly positive	1	4	13	18
Negative to weakly positive	14	11	2	27
Total	15	15	15	45

p value <0.001 (significant)

DISCUSSION

Activating mutations in BRAF have been reported in 5–15% of colorectal carcinomas (CRC). The most common BRAF mutation corresponds to a T>A transversion at position 1799, resulting in the substitution of Valine by Glutamate at position 600 of the protein.[13] This mutation results in an enhanced activity of the kinase domain, stimulating cell division, cellular survival, proliferation, differentiation, and inhibition apoptosis.[9]

Affolter et al used antibody VE1 for immunohistochemistry to detect BRAF V600E mutations in CRC and there was 100% sensitivity

and specificity relative to pyrosequencing.[12] We used this technique as an alternative to molecular methods for detecting of the BRAF V600E mutation.

In our study we scored BRAF V600E immunoexpression by multiplying the percentage and the intensity score and then we classified these score into negative immunoexpression, weakly positive immunoexpression, moderately positive immunoexpression and strongly positive immunoexpression. In some journals a tumor was considered immunopositive for BRAFV600E if it displayed a staining intensity of moderately positive or strongly positive irrespective of the number of tumor cells stained, but some journals scored by multiplying the percentage of positive cells with staining intensity.[12,14,15]

Interestingly, we observed BRAF mutation were present in 18 cases (11 cases of 45 cases with moderately positive immunoexpression and 7 cases of 45 cases with strongly positive immunoexpression) of all samples; while studies performed by Marin et al., Wilmott et al. categorized weakly positive BRAF V600E immunoexpression as negative result.[15,16] The highest frequencies positive BRAF V600E immunoexpression were seen in Dukes' stage C.

Positive BRAF V600E immunoexpression were more frequently seen in Dukes' stage C CRC explained clearly that BRAF V600E play an important role in pathogenesis colorectal carcinoma. The Ras/Raf/MEK/ERK mitogen-activated protein kinase (MAPK) pathway is a critical signal transduction pathway involved in many cancers, including colorectal cancer. The BRAF oncogene, which is immediately downstream of Ras in the MAPK cascade. The MAPK signalling cascade functions immediately downstream of cell surface receptors and cytoplasmic signalling proteins, relaying extracellular signals to transcriptional regulation of fundamental cellular functions such as growth, survival, proliferation, differentiation and migration. This pathway is deregulated in about 30% of all human cancers and can be activated by mutations in oncogenes such as KRAS and BRAF, or activation of upstream receptor tyrosine kinases such as EGFR.[8,17] Mutations in the kinase domain of the BRAF gene can lead to constitutive activation of the enzyme, resulting in dysregulated downstream signaling via MEK and ERK, excessive cell proliferation, and survival independent of external cellular signals.[18]

In this study positive BRAF V600E immunoexpression was more frequent in colorectal carcinoma Dukes' stage C (14/15). This result was significant (P<0.000) using Chi-Square test analysis. These result was similar with the resluts of study don by Busby et al which used the mismatch ligation assay (MLA) technique to detect the BRAF mutant tumours, found that four of the five BRAF mutant tumours were Dukes' stage C, with the other being Dukes' D, and the results were significantly more likely to be stage C or D, rather than A or B, than those without such a mutation (5/5v15/38 primary cancers).[19]

Ogino et al. studied BRAF mutation effect on survival and treatment efficacy in patients with stage III (Dukes' stage C) colon cancer and they confirmed that BRAF-mutated CRC is associated with inferior survival in stage III (Dukes' stage C) colon cancer.[20] This data showed that the colorectal cancer with BRAF mutation have Dukes'stage C and poorer prognosis and lower response than BRAF type. Result of this study showed that BRAF V600E immunoexpression was most frequent in aggresive colon cancer. Therefore, Dukes stage B CRCs need BRAF V600E immunoexpression examination. If Dukes stage B CRCs had positive BRAF V600E immunoexpression, it can be used as a guideline for giving adjuvant chemotherapy.

Farin a-Sarasqueta et al. assessed the effect of the V600E BRAF mutation, in stage II (Dukes' stage B) and stage III (Dukes' stage C) colon cancer patients. The carriage of the mutation accounts for a significantly higher risk of dying of cancer-related causes, independently of other factors like age, sex, location of the tumor, MSI status, KRAS mutational status, differentiation grade, T stage and N stage.[7]

The epidermal growth factor receptor (EGFR), represents an important target for cancer treatment because its activation stimulates key processes involved in tumor

growth and progression, including proliferation, angiogenesis, invasion, and metastasis. EGFR inhibitors-monoclonal antibodies targeting the extracellular domain and small-molecule tyrosine kinase inhibitors have been extensively studied in metastatic colorectal cancer. The first EGFR monoclonal antibody to be approved for clinical use for metastatic colorectal cancer (Dukes's stage C) has been evaluated primarily in combination with chemotherapy but also as monotherapy.[21]

Positive EGFR protein expression, as determined by immunohistochemistry, was initially selected as an entry criterion for studies evaluating EGFR inhibitors with assumption that sensitivity to such agents was associated with EGFR expression. However, a large body of evidence from patients who were treated with monoclonal antibodies for metastatic colorectal cancer or tyrosine kinase inhibitors for other solid tumors indicates that this biomarker is poorly associated with response to EGFR inhibitors in the clinical setting.[21] In a recent study, the efficacy of antibody therapy against the epidermal growth factor receptor for wild-type–KRAS tumors appeared to be restricted to wild-type–BRAF tumors.[22] Immunoexpression of BRAF V600E can be used to know the efficacy of antibody therapy against the epidermal growth factor receptor.

Signaling through the EGFR and its downstream pathways presents numerous potential targets for treating patients with cancer. Many clinical trials are underway using agents MAP kinase signaling, including BRAF.[9] The clinical development of BRAF inhibitors in colorectal cancer carrying the BRAF V600E mutation, the enriched population, will be challenging due to the low BRAF V600E mutation rate in this tumour type.[8] Efficacious targeted therapy would expand the indications for BRAF gene testing.[9] Immunohistochemical detection of BRAF V600E mutation is a fast, inexpensive technique and seems to have a very good specificity.[16] As immunohistochemistry is cheaper and results are easily obtained within 48 hours, it could be used as the first step for BRAF mutational status.[23] BRAF V600E immunohistochemistry could be used as first screening for efficacy of BRAF V600E targeted therapy.

CONCLUSION

The immunoexpression of BRAF V600E is mostly present in Dukes' stage C CRC.

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